The vulnerable plaque: From plaque instability towards thrombus instability
Li, Xiaofei

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Neutrophils, neutrophil extracellular traps and interleukin-17 associate with the organisation of thrombi in acute myocardial infarction

Onno J de Boer*, Xiaofei Li*, Peter Teeling*, Claire Mackaay*, Hanneke J. Ploegmakers, Chris M van der Loos*, Mat J Daemen*, Robbert J de Winter* and Allard C van der Wal*

From the departments of Pathology (*) and Cardiology(#), Academic Medical Center, Meibergdreef 9, 1105 AZ Amsterdam, the Netherlands

Abstract

Background
Neutrophils are important cellular sources of Interleukin 17A and –F. Moreover, upon activation neutrophils are able to excrete chromatin embedded with components from their cytoplasmic granules to form ‘neutrophil extracellular traps’ (NETs). Recent studies suggested that NETs contribute to thrombosis by promoting fibrin deposition and platelet aggregation. IL17A may also promote thrombosis by enhancing platelet aggregation. In the present study we investigated the presence of neutrophils, NETs and IL17A and -F in coronary thrombosisuction specimens obtained from patients after acute myocardial infarction.

Methods & Results
Neutrophils and NETs were identified using histochemical (H&E, Feulgen procedure) and immunohistochemical stainings (Histone H1, myeloperoxidase, neutrophil elastase) in 15 fresh, 15 lytic and 15 organized thrombi. The presence and distribution of IL17A and –F was studied using (immuno)histochemical doublestaining and spectral image analysis, rtPCR and Western Blot. High numbers of neutrophils are present (10-30% of the thrombus mass) in fresh and lytic, but not in organized thrombus. NETs were frequently observed in fresh (4/15) and lytic (12/15), but never in organized thrombus specimens. Doublestaining combining the Feulgen reaction with Histone-H1, MPO or neutrophil elastase confirmed colocalization with DNA. Cytoplasmatic IL17A/F staining was found in the majority of the neutrophils, extracellularly and in NETs. Western blotting confirmed the presence of IL17A and IL17F in thrombus specimens.

Conclusion
A large burden of neutrophils, neutrophil extracellular traps and IL17A and –F are important constituents of fresh and lytic thrombus after acute myocardial infarction. The specific colocalization of these indicates a role during thrombus stabilization and growth.
Neutrophils, NETs and IL-17 in coronary thrombi of STEMI patients

Introduction

ST elevation type of myocardial infarction (STEMI) is in most cases initiated by the rupture of a coronary atherosclerotic plaque that is followed by the thrombotic occlusion of the vessel(1). Recent histopathological studies on coronary thrombectomy specimens obtained from STEMI patients have shown that the occluding thrombus frequently shows features of decay or even organization, which is illustrated by the ingrowth of smooth muscle cells and blood vessels(2). This implicates that in these patients the initiating stimulus, plaque-rupture or erosion occurred at least several days up to a week before the onset of clinical symptoms(2). Moreover, we have found that thrombus organization at the time of thrombus aspiration is an independent risk factor for future cardiovascular death in STEMI patients (3). Pathophysiological, this process of propagation and eventual organization of a coronary thrombus is far from understood.

Arterial thrombus contains, apart from fibrin and platelets also neutrophils as the most prominent vital cellular component(2;4). Recent evidence has shown that neutrophils perform their inflammatory effector functions not only by the secretion of inflammatory mediators or phagocytosis, but also through also through the formation of so called Neutrophil Extracellular Traps (NETs). NETs are extracellular networks consisting of chromatin fibers (DNA and histone proteins) covered with enzymes from the contents of the cytoplasmatic granules of these cells(5). Upon activation, NETs can be secreted by a subpopulation of viable neutrophils, or alternatively, formed as the result of a recently identified, new type (Caspase independent) of cell death, NETosis(6). Neutrophil-derived NETs, covered with myeloperoxidase (MPO), elastase or Calprotectin play an important role during the inflammatory response against bacteria(5;7-9). In addition, it was shown recently that NETs are probably also operative in the process of thrombus formation, at least in veins(10-12). In this respect it is also interesting to note that platelets, abundantly present in thrombus material, promote NET formation(13;14) IL17A and -F belong to a recently identified cytokine family which members are believed to play an important role during inflammation and autoimmunity(15). A novel subset of T cells, designated Th17 cells are considered as important cellular source of IL17A and –F, but several other inflammatory cells are able to produce these cytokines as well. Recently we showed that IL17A and F are expressed by mast cells and neutrophils in human atherosclerotic plaques, and the latter mostly in ruptured plaques((16). Interestingly, there are also recent data showing that IL17 may play a role during thrombosis by augmenting platelet aggregation(17).
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Based on these considerations we hypothesized that NETs are coated with IL-17 and these NETs and IL-17 are involved in the process of intracoronary thrombus formation in patients with acute myocardial infarction (AMI). By means of histological grading of the age of the thrombi we investigated whether IL-17 and/or NETs can be found in the different stages of coronary thrombus evolution, and whether IL-17 and NETs colocalize.

Materials & Methods
Specimens
In the present study a total of 62 thrombi were used. For the histopathological part of the study, we retrieved 45 specimens (15 fresh, 15 lytic and 15 organized thrombi) from our archive of collected thrombectomy specimens, which contains over 2000 histologically classified thrombus samples. The histopathological classification of thrombi that we used is based on the age of the thrombus material and has been described before(2;18;19): 1: fresh thrombus (less than 1 day old): completely composed of layered patterns of platelets, fibrin, erythrocytes and intact granulocytes; 2: lytic thrombus (between 1 and 5 days old) was characterized by areas of colliquation necrosis and karyorrhexis of granulocytes; 3: organized thrombus (more than 5 days old) with areas with ingrowth of smooth muscle cells, with or without depositions of young connective tissue and ingrowth of capillary vessels. Thrombus material with a heterogeneous composition was graded according to the age of the oldest components. A total of 17 tissue samples were snap frozen in liquid nitrogen and used for RNA (n=14) or protein (n=3) extraction. Because the amount of tissue obtained during thrombectomy procedures is very small, and all the material was used RNA or protein isolation, it was not possible to perform histopathology and subsequent staging on these samples to evaluate the degree of organization. Upon retrieval from the patient, thrombectomy specimens were either immediately transferred to the formalin solution (in the catheterization room) or, when they were frozen, transferred to the pathology lab, where they were snap frozen in liquid nitrogen. In case of the latter, the interval between retrieval and freezing was always less then 10 minutes. The study confirms with the principles outlined in the Declaration of Helsinki. The medical ethical review board of the Academic Medical Center granted a waiver for informed consent because only leftover tissue of normal clinical procedures was used in this study. All acquired data were analyzed anonymously.
Histochemistry & Immunohistochemistry
All specimens used for histochemistry and immunohistochemistry were washed in PBS, formalin fixed and paraffin embedded using standard procedures. For marking nuclear elements, hematoxylin (as part of H&E stains), Feulgen reaction and DAPI were prepared using standard laboratory procedures. For immunohistochemistry, the following primary antibodies were used: polyclonal rabbit anti myeloperoxidase (MPO, Dako, Glostrup, Denmark), mouse anti neutrophil elastase (clone NP57, Dako), mouse anti histone H1 and mastcell tryptase clone G3 (Milipore, Billenica, CA, USA) goat anti IL17A (R&D Systems, Abington, UK). Immunohistochemical stainings were performed as previously described(20). Immunohistochemical stainings were also combined with the Feulgen reaction to investigate colocalization with DNA. In short, sections were dewaxed and rehydrated, followed by antigen retrieval using Tris EDTA (pH = 9.0) for 20 minutes at 98°C. After washing, the sections were subsequently incubated with appropriate dilutions of the different primary antibodies. After the goat anti IL17 incubation rabbit anti goat Ig (Southern Biotech, Birmingham, AL, USA) served as a bridge reagent for the next step with alkaline phosphatase (AP) anti rabbit Ig polymer (Immunologic, Duiwen, The Netherlands). Depending on the origin of the primary antibodies, sections were incubated with appropriate AP conjugated anti mouse- or anti rabbit-Ig polymer (Immunologic). AP activity was visualized with Vector Blue (Vector Labs, Burlingame, Ca, USA). Following immunohistochemical staining including AP visualization, a Feulgen counterstain was performed to investigate colocalization with DNA. Sections were pretreated with HCl (1N), washed with tap water and incubated with Schiff reagent (Merck, Darmstadt, Germany), washed and finally treated with 5% potassium metabisulphite. For investigating colocalization of IL17A/F with MPO and tryptase a sequential double staining was performed(16;20) with IL17 in blue (Vector blue) and MPO or tryptase in red (Vector red). All sections were organically mounted with Vecta Mount (Vector Labs).
Negative controls (sections in which the specific primary antibody had been omitted or had been replaced by isotype and concentration matched antibodies) were always included. Because the goat polyclonal goat anti IL17A antibody is known to cross react with IL17F (not only according to the supplier, but also in our experience), results that were obtained with this antibody will be further designated in this paper as IL17A/F.

Quantification of Neutrophil Extracellular Traps in thrombectomies
The presence or absence of NETs in thrombus tissue was scored by two observers (OJdB, XL), and disagreements were resolved by consensus. For this, sections with H&E, Feulgen and H1 stainings were available for all specimens.
Spectral Imaging

Analysis of double stained slides was performed with spectral imaging using the Nuance VIS-FL Multispectral Imaging System (Caliper Sciences, Hopkinton, MA, USA) as previously described (20;21). Data sets were acquired from 420-720 nm at 20 nm intervals. A spectral library composed of single Vector Blue, single Vector Red and Feulgen reaction was applied to unmix the double stainings into individual components. Using the Nuance software version 3.0, exclusive images of colocalization as well as fluorescent-like pseudo colors were created.

RNA isolation and rtPCR

RNA was extracted from 14 freshly frozen thrombectomies using the Trizol method as previously described (16). cDNA was synthesized from 5μg total RNA using oligo dT as primer and M-MLV reverse transcriptase (Invitrogen). Primers for specific for IL17A (5’-GGAATCTCCACCGCAATGA-3’ and 5’-AGAGCTCTTAGGCGCA-CATGCT), IL17F (5’-GCCAGCCATGGTCAAGTA-3’ and 5’-AGTGTAATTC-CAGGGGAGGT-3’) and β-actin (5’-CCTTCCTGCGGATGGAT-3’ and 5’-GCTCAGGGAGCAATGATCT-3’) were designed using primer3 sofware (22). cDNA was amplified by PCR as previously described (16), electrophoresed on a 1% agarose gel, and visualized using ethidium bromide staining. Positive controls (in vitro activated peripheral blood mononuclear cells (16), and negative controls, a mock PCR (with water instead of cDNA) were always included.

Western Blotting

Three entire freshly frozen samples were used for IL17A and IL17F protein detection by means of Western blot analysis. Thrombectomies were treated with lysis buffer (10mM Tris HCl (pH 8), 50mM NaCl, 5mM EDTA, 1% NP40, 10% glycerol). Lysates, positive- and negative controls (rIL17A and rIL17F) were separated by SDS-PAGE under non-reducing conditions, and transferred to methanol activated PVDF membranes (Immobilon P, Millipore). After blotting, the membranes were blocked in blocking buffer containing 5% non fat dried milk in 0.1% Tween20 for 1 hour at room temperature and incubated overnight at 4°C with the primary antibodies (polyclonal rabbit anti IL17A or mouse monoclonal anti IL17F (R&D Systems). Next membranes were washed and sequentially incubated with rabbit anti goat Ig (Southern Biotech) and HRP conjugated swine anti rabbit Ig (Dako). Finally, antibody labelled proteins were visualized using ECL+ (Roche, Almere, the Netherlands) and the LAS-3000 imaging system (FujiFilm, Düsseldorf, Germany).
Statistics
Statistical differences between variables were calculated using 2 tailed Fischer's exact test. P<0.05 was considered statistically significant.

Results
Neutrophils and NETs in coronary thrombus
All the thrombus samples that were histologically graded as either fresh or lytic thrombus contained large amounts of neutrophils (up to 10-30 % of the total thrombus area in the tissue section). Representative examples of a fresh and lytic thrombus are illustrated in figures 1A and 1B, respectively. In organized thrombi on the other hand, neutrophils are very scarce or completely absent (figure 1C).

H&E staining and Feulgen sreaction clearly revealed the presence of NETs in a part of the thrombectomy specimens as thin extracellular fibers in various sizes (see figure 1D and E). Immunohistochemical staining for H1 (figure 1F) further confirmed the presence of NETs in these specimens. H&E stains and Feulgen reaction provided the most detailed images of NETs, and were used for the semi-quantitative grading for the presence of NETs in the thrombus samples.

Figure 1. H&E stainings of thrombi at different stages of thrombus organization (A-C) and the presence of intracoronary NETs visualized using histological and immunohistological staining (D-F). A: fresh thrombus. Note the large amount neutrophils in this section. Bar = 50µm. B: High power magnification of a lytic thrombus. Note the neutrophils and leucocytoclasia, Bar = 20µm. C: Organized thrombus. Note the absence of neutrophils. The red colored spindle shaped structures are collagen fibers produced by myofibroblasts, Bar = 100µm. D: H&E staining, showing a lytic thrombus area with neutrophils and NETs, bordered by viable red blood cells in the upper left and bottom right corner. Bar = 50µm E: Feugen staining (DNA stained in pink) of the same section as in (D); F: Same section, immunohistochemical staining for histone H1; G: Bar graph showing the percentage of fresh, lytic and organized thrombi where NETs were encountered. *: P < 0.05.
presence in all thrombi. NETs were not homogeneously distributed over the thrombus specimen, but were present as focal hotspots of varying size. The total area of NETs (when present) was variable between specimens, but always less then 10% of the total thrombus area.

NETs were most frequently found in lytic thrombus specimens: in 12 of the 15 specimens (80%) NETs were encountered (figure 1G). In 4/15 (27%) fresh thrombus specimens NETs were encountered (p<0.05 compared to lytic thrombus). In organized thrombus tissue NETs were never observed.

**Colocalisation of Feulgen reaction with MPO and neutrophil elastase NETs**

Additional histochemical and immunohistochemical stainings were performed to further evaluate the NETs. First, we stained the sections using the Feulgen reaction, which is (in contrast to haematoxylin) specific for DNA(23). A representative example of an NET in a thrombectomy stained with Feulgen reaction is illustrated in figure 2A. Figure 2B shows a DAPI (fluorescence) staining the same thrombectomy specimen. The thin and thread like structures that are characteristic for NETs can be appreciated in both stains.

Next immunohistochemistry combined with the Feulgen reaction was performed and the results were analyzed by spectral imaging. In figure 2C the Histone H1/Feulgen combination (in blue and pink, respectively) is illustrated. Figure 2D and 2E show the tissue distribution of Histone H1 in and DNA in green and red fluorescent-like pseudo colors, respectively, while in figure 2F colocalization is illustrated in yellow. Non-colocalized staining is depicted in grey. These pictures clearly show the overlap in expression of the Feulgen reaction and the immunohistochemical localization of Histone H1. Similar double staining sets were created for a double staining with Feulgen and neutrophil MPO (figure 2 K-N) and Feulgen with neutrophil elastase (figure 2 O-R). All these double stainings and colocalization studies confirmed the colocalisation of MPO and elastase by NETs in human coronary thrombus material, as proof that these structures are indeed NETs.

As additional controls we performed a DNase treatment after the immunostaining, but before the Feulgen reaction. In figure 2 G-J such a control, which was stained for Histone H1 is illustrated. It can be appreciated that the Feulgen reaction disappeared after DNase treatment, and almost all colocalization disappeared.
Figure 2. Histochemical and immunohistochemical characterization of NETs in intracoronary thrombi. A: Feulgen reaction. DNA stained in pink. B: DAPI staining. C-F: Feulgen reaction combined with immunohistochemistry. C: original RGB image showing H1 in blue and Feulgen in pink; D,E: Histone H1 and Feulgen reaction component images, respectively, after spectral unmixing in fluorescent-like pseudo-colors; F: spectral imaging analysis showing an exclusive image of Histone H1 and Feulgen reaction co-localisation (yellow) against a grey background of both Histone H1 and Feulgen. Similar analysis is applied to G-J, K-N and O-R. G-J: Feulgen reaction combined with immunohistochemistry anti Histone H1. After the immunohistochemical staining for H1 sections were treated with pepsin/DNase. Note the absence of colocalization after DNase treatment; K-N: Feulgen reaction combined with myeloperoxidase; O-R: Feulgen reaction combined with neutrophil elastase. Bar = 25μm (all images)
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Immunolocalization of IL17A/F in thrombus specimens
We next investigated the expression and distribution of IL17A/F in human thrombi. Figure 3A shows a low power magnification with an overview of a thrombus, stained for IL17A/F (in blue) and MPO (red). In this picture it can be appreciated that high numbers of IL17A/F positive neutrophils (all the purple cells) are present in such a thrombus. In figure 3B a detail of this section is illustrated, showing double stained IL17A/F positive neutrophils, and a few neutrophils that do not co-express IL17A/F (red cells). In figure 3C-3F, 3G-3J, and 3K–3N the IL17A/F – MPO double staining was confirmed using spectral imaging analysis. In figure 3C the original staining is illustrated, while figure 3B and 3C show the same immunodouble stained section of which both colours are unmixed. In figure 3F colocalization is illustrated. From these images it can be appreciated that the majority neutrophils co-express IL17A/F. However, occasionally (< 10%) IL17A/F negative neutrophils were encountered. Extracellular granules positive for IL17A/F were also occasionally seen in the direct neighborhood of neutrophils. Figure 3G-J show a similar double staining with IL17A/F and MPO of neutrophils in adherent blood (‘stasis blood’) which can be frequently encountered amidst thrombus fragments. It appears that also in this micro-environment the majority of the neutrophils co-express IL17A/F but there is a subtle difference in expression pattern: in contrast to the neutrophils in the thrombus, these neutrophils show a cytoplasmic staining located in the periphery of the cell. In organized thrombus we did not observe IL17A/F expression. Mast cells are also able to express IL17, and therefore we also performed immunohistochemical doublestainings with IL17A/F and tryptase. However, mast cells (thus also tryptase+/IL17A/F+ cells) were never encountered in any of the thrombectomy specimens (data not shown).

When combining the Feulgen reaction with immunohistochemistry against IL17A/F we found that NETs in fresh and lytic thrombi also express IL17A/F (figure 3K-N), but the amount of colocalization was less compared to MPO and elastase.

Given the cross reactivity of the polyclonal antibody between IL17A and IL17F, we further analyzed the presence of both these cytokines in human thrombus using rtPCR and Western Blot. The rtPCR results are illustrated in figure 4a. Interestingly, IL17A mRNA was not detectable in the thrombectomies under investigation. Weak bands corresponding with IL17F mRNA were observed 4/14 specimens (figure 4A).

Western blot analysis was performed on 3 thrombectomy specimens. Figure 4B shows that IL17A protein was detectable in the positive control (rIL17A) and all specimens under investigation. However, this figure also shows that this antibody shows a very faint band with rIL17F that we used as negative control, which confirms that this antibody shows some cross reaction with IL17F. In addition, IL17F was also clearly detectable in all specimens under investigation, but this antibody did not cross react with rIL17A (figure 4C).
Figure 3. Colocalization of IL-17 with Neutrophils and NETs in thrombus. A: Overview (low power magnification) and detail (B) of a IL-17 (blue) and MPO (red) double staining. Note that almost all neutrophils are IL17+ (purple), and only few IL-17 neutrophils (red) are present. Asterisk (*) indicates adhered (stasis) blood, where also IL-17+ neutrophils are present. C-F: Spectral image analysis on a double staining with MPO and IL-17 of a lytic thrombus. C: original RGB image showing MPO in red and IL-17A/F in blue. D, E: IL-17 and MPO after spectral unmixing. F: colocalization. Note again that most neutrophils co-express IL-17A/F. G-J: same staining on neutrophils in adhered blood. Note the colocalization of IL-17A and MPO, and the altered staining pattern when compared to (D). K-N: Feulgen reaction combined with immunohistochemistry against IL-17A. K: original RGB image showing Feulgen in pink and IL-17A in blue. L, M: IL-17A and Feulgen after spectral unmixing, respectively, N: colocalization in yellow. Bar = 200μm (A) or 25μm (C-N).
Neutrophils form an integral part of the vital (cellular compartment) of coronary thrombus(4). In the present study we found that thrombi derived from STEMI patients shortly after the onset of the acute event contain on the average 10-30% of granulocytes as their component makeup when they are fresh or only a few days old. In older stages of thrombus evolution, characterized by tissue organization, they are virtually absent. NETs, which are recently described activation products of neutrophils, could be found focally in older thrombi with lytic changes, but also, albeit to a lesser extent, in thrombus that we considered histologically as fresh. Moreover, these thrombus fragments contained IL17A and IL17F where it was stored in neutrophils but also extracellularly attached to the NETs.

There is mounting evidence that NETs play a role during thrombosis. NETs promote fibrin deposition, red blood cell recruitment and provide a stimulus and scaffold for the formation of fibrin networks(12). In addition, in an experimental model of deep vein thrombosis in baboons it has been shown that NETs are present in thrombi in vivo(12). In a mouse model of venous thrombosis it has been shown that plasma DNA levels increase after the induction of experimental thrombosis and interestingly, prior DNase infusion in these animals prevented the development of venous thrombi(11). Recently Megens et al. showed the presence of NETs in carotid arteries of ApoE−/−.

Figure 4. A: rtPCR of 14 thrombectomy specimens for IL-17A and IL-17F. IL-17A mRNA is undetectable, while weak expression of IL-17F RNA is occasionally observed. B and C: Western blotting of 3 thrombectomies for IL-17A and IL-17F, respectively. Note the expression of both IL-17A and IL-17F protein in all specimens under investigation. A= rIL17A, F = rIL17F.
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mice, suggesting that activated neutrophils and NETs may contribute to the initiation of atherosclerotic disease (24). In the present study we show that NETs are abundantly present in human intracoronary thrombi. Because these NETs from scaffolds for fibrin threads, which in their turn are important for platelet aggregation, it is fair to conclude that neutrophils contribute to thrombus growth and stabilization by the formation of NETs as we found in this study.

Not only NETs, but also cytokine IL17 can be linked to thrombus stabilization. Platelets express the receptor for IL17A and -F (IL17RA) and incubation of platelets with IL17A promotes platelet aggregation (17). IL17, associated with NETs is therefore in the perfect position to promote the deposition and aggregation of platelets on NETs, thus promoting the stabilization of thrombus. Moreover, IL17A promotes angiogenesis (25), which contributes to later stages of thrombus organization.

Not all plaque ruptures in the coronary arteries lead to complete thrombotic occlusion and consequent myocardial infarction. Autopsy studies have shown so called healed plaque ruptures, interpreted as clinically silent plaque complications, in many high grade stenosing lesions of coronary arteries (18). Moreover, we have shown that thrombus of patients with STEMI frequently contains older parts which must have been formed days or sometimes weeks before the onset of the event (2). This implicates that the formation, stabilization and growth of an intracoronary thrombus is crucial process which determines the clinical outcome of a plaque rupture. The formation of NETs, or conversely, the degradation of NETs can be important in this sequence of events. NETs can be formed in response to inflammatory cytokines like IL-8 and TNF-α (26). Circulating IL-8 or TNF-α, or locally produced by inflammatory cells (macrophages) in the recently ruptured atherosclerotic plaque may promote NETosis, leading to thrombus growth and stabilization. On the other hand, circulating DNases in plasma are able to break down NETs, and thus may prevent thrombus growth and stabilization. In this respect it is interesting to note that the levels of DNases are known to fluctuate between individuals (27). Data on eventual levels of circulating DNases and the risk for developing acute myocardial infarction are at present not available.

NETs were observed not only in thrombi with lytic changes, but also in a few specimens (4/15) that appeared histologically as fresh. NETs are formed as the result of local neutrophil activation, which also may lead to karyorrhexis and colliquation necrosis. It can therefore be assumed that the formation of NETs precedes the lytic changes that can be observed histologically, and occur very early in the process of thrombus dissolution.

Not only neutrophils are able to generate these extracellular traps, but also mast
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cells are able to form this type of structures, designated mast cell extracellular traps (MCETs)(28). Mast cells are also able to produce IL17A and -F cytokines, and therefore in theory, these cells could also from extracellular traps with IL17 in thrombus. We also looked for IL17A/F+ mast cells this series of thrombectomies using a double staining for IL17A/F and tryptase, but these cells were never encountered, and all IL17A/F positive cells were neutrophils. Therefore it is fair to state that ‘extracellular traps’ we observed in human thrombus are neutrophil-, and not mastcell- derived.

An interesting ‘discrepancy’ in our results was the clear and strong expression of IL17A and F protein in human thrombus specimens (as observed with immunohistochemistry and Western blots), while the rtPCR showed no (IL17A), or occasional weak (IL17F) expression of mRNA of these cytokines. These data show that most neutrophil IL17A and –F is already present in the cytoplasmic granules of the neutrophils before these were trapped in the thrombus. In addition, these data show that the production of IL17F, but not IL17A still continues, although at relative low levels, in the microenvironment of the thrombus.

In conclusion, neutrophils, neutrophil extracellular traps and IL17A and –F are important constituents of fresh and lytic thrombus after acute myocardial infarction. The specific colocalization of these in fresh and lytic thrombi suggests that they may play an important role during thrombus stabilization and growth.
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